

## Comment

## Free radicals run in lizard families: a mitochondrial uncoupling phenomenon or not?

Olsson and co-authors have recently published interesting papers on oxidative stress in lizards. The aim is to understand the mechanisms defining ageing rates in free-ranging animals, particularly by looking at mitochondrial production of reactive oxygen species (ROS). Indeed, ROS, and more specifically superoxide, are chronically formed at different sites of the electron transport chain (ETC) during oxidative phosphorylation. The induced potential oxidative stress has been proposed by the 'free radical theory of ageing' to result in accumulation of macromolecule damage that causes ageing (Beckman & Ames 1998). Olsson's studies report that differences in levels of ROS production exist among families and suggest that these differences can be driven by natural selection. In their recent paper (Olsson *et al.* 2008), the authors demonstrated that ROS production levels are heritable, a relevant result in understanding the physiological basis of life-history traits.

This study is based on an innovative approach using cell biology methods to tackle a question relating to evolutionary biology. To evaluate ROS production within the mitochondria, superoxide production was measured by flow cytometry. Interestingly, mitochondrial ROS production was modified using carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a proton-ionophore uncoupler that reduces the mitochondrial membrane potential by allowing the protons to cross back through the inner membrane by a pathway other than ATP synthase. CCCP is therefore accelerating the working rate of the ETC, resulting in an enhanced consumption of oxygen for an identical mitochondrial electrochemical gradient (uncoupling effect). The authors argue that 'CCCP administration mildly uncouples mitochondria, which increases electron flow through the ETC [...] may lead to increased ROS production...'. Accordingly, they found an increased ROS production after CCCP treatment, which is heritable in lizard families. In this paper the authors conclude that using their 'uncoupled superoxide measurement, the extremely high heritability of superoxide in combination with CCCP uncoupling, suggests strong family effects on how this molecule affects the proton gradient' (Olsson *et al.* 2008).

However, this association between an uncoupled state of mitochondria and a rise in ROS production contradicts recent ideas about mitochondrial uncoupling, ROS and longevity (Brand 2000). When the ETC and its electron flow is slowed down, the formation of superoxide is promoted by the one-electron reduction of oxygen molecules mainly by complexes I and III (Ricquier & Bouillaud 2000). The 'uncoupling to survive' hypothesis proposed that an increased electron transfer through the ETC by mild uncoupling inhibits superoxide formation by shortening the half-life of reduced coenzymes such as ubiquinone (QH<sup>•</sup>; figure 1), a potent ROS initiator (Skulachev 1996). Accordingly, production of ROS after the addition of an uncoupler on isolated mitochondria is undetectable (Boveris & Chance 1973).

Besides the fact that CCCP may have a different effect on entire cells from isolated mitochondria through plasma membrane depolarization (Lichtshtein *et al.* 1979), we believe that there is a misunderstanding of what is actually happening at the mitochondrial level when lizard cells have been treated with CCCP. None of the references given by the authors explain the CCCP-induced increase of ROS production by the uncoupling activity *per se*, but rather emphasize a negative impact on antioxidant defences (thiol groups) or a stimulation of calcium-dependent ROS production. As pointed out by Olsson, CCCP impact is dose dependent, leading either to a decrease or an increase in ROS production. It has been shown in mammalian cells that use of CCCP at more than 10  $\mu$ M induces a rise in ROS production (Mozo *et al.* 2006). This biphasic effect of CCCP on ROS formation tallies with its effect on oxygen consumption: using CCCP to record the maximal (fully uncoupled) rate of respiration of mitochondria needs an optimal concentration to be found. When CCCP concentration increases above this optimum, respiration starts to be inhibited. A likely explanation is that the uncoupler (a lipophilic molecule) disturbs the ETC at the level of coenzyme Q (another lipophilic molecule). Moreover, when the prevention of ROS formation is referred to as 'mild uncoupling', the term 'mild' is opposed to 'full' uncoupling which would virtually abolish mitochondrial ATP production. This suggests that the sensitivity of lizard cells to enhanced CCCP ROS production is not linked to 'how the proton gradient is affected' (as stated by the authors), but rather to two non-exclusive possibilities: inter-familial variability of (i) the adverse impact of CCCP treatment on antioxidant defences, or (ii) resistance of respiratory chain complexes to CCCP inhibitory impact.

These considerations may be crucial to the authors' interpretation since they studied the mechanisms that shape the heritability of mitochondrial oxidative stress. As they rightly concluded in their paper, 'an important way towards understanding the evolution of net ROS levels may be to investigate heritability and genetic correlations between ROS and ETC traits'. While this is an accurate aim, they should not miss the relevant target.

The accompanying reply can be viewed on page 345 or at <http://dx.doi.org/doi:10.1098/rsbl.2009.0115>.

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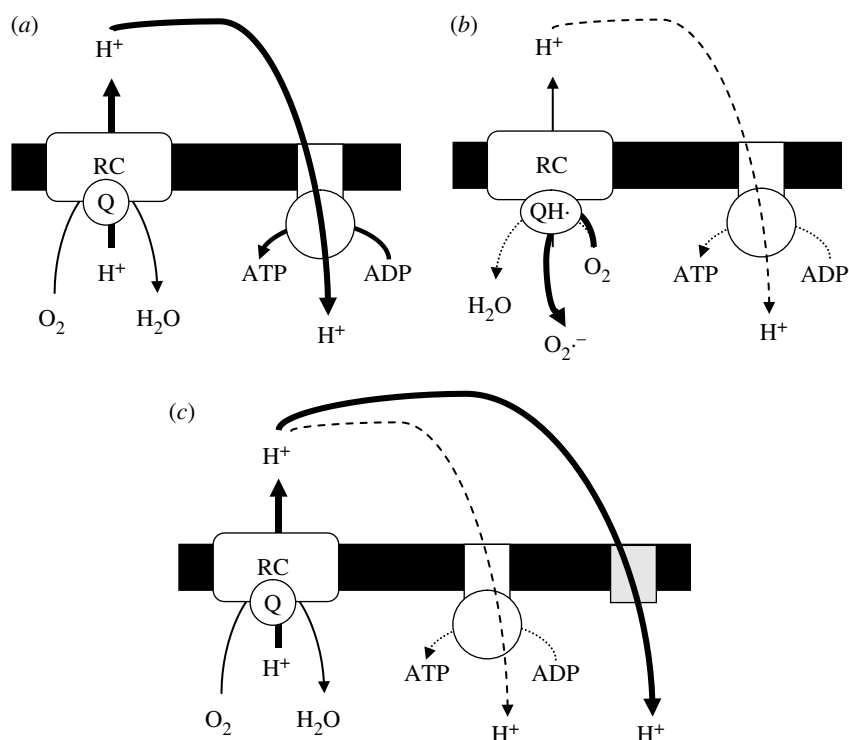


Figure 1. Regulation of ROS production by the respiratory chain (RC). (a) When respiration is coupled to ATP production, electrons go easily through the RC and react with the final acceptor ( $O_2$ ) to produce  $H_2O$ . (b) As soon as the ATP production is reduced, the electron flow is slowed down and reduced coenzymes as ubiquinone ( $QH\cdot$ ) may react directly with oxygen to produce superoxide. (c) A mild uncoupling induced by the opening of a second door through the inner mitochondrial membrane allows protons to return to the mitochondrial matrix, which enhances respiration and restores the normal half-life of reduced coenzymes, thereby reducing the risks of superoxide formation.

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